A specific subdomain in ϕ29 DNA polymerase confers both processivity and strand-displacement capacity

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Recent crystallographic studies of ϕ29 DNA polymerase have provided structural insights into its strand displacement and processivity. A specific insertion named terminal protein region 2 (TPR2), present only in protein-primed DNA polymerases, together with the exonuclease, thumb, and palm subdomains, forms two tori capable of interacting with DNA. To analyze the functional role of this insertion, we constructed a ϕ29 DNA polymerase deletion mutant lacking TPR2 amino acid residues Asp-398 to Glu-420. Biochemical analysis of the mutant DNA polymerase indicates that its DNA-binding capacity is diminished, drastically decreasing its processivity. In addition, removal of the TPR2 insertion abolishes the intrinsic capacity of ϕ29 DNA polymerase to perform strand displacement coupled to DNA synthesis. Therefore, the biochemical results described here directly demonstrate that TPR2 plays a critical role in strand displacement and processivity.

protein-primed replication | terminal protein region | helicase-like activity | DNA-binding stability

DNA replication is a complex multistep process that involves a wide range of proteins and enzymatic activities (1, 2). DNA synthetic activity is provided by DNA polymerases that add nucleotides to the 3′-OH end of a primer strand guided by base pairing with the template strand. Polymerases involved in DNA replication are referred to as replicases to distinguish them from other DNA polymerases whose synthetic activities play a role in processes such as DNA repair or recombination. In most DNA replication systems, replication fork movement along the duplex DNA requires an unwinding activity to separate the strands as replication progresses (1, 2). Generally, such activity is not intrinsic to the replicase but is provided either by monomeric or multimeric enzymes called helicases, which melt the dsDNA in an ATP-dependent fashion. In addition, the intrinsic processivity (number of nucleotides incorporated per single DNA polymerase/DNA-binding event) of most replicases is not high enough to account for the replication of an entire genome, and therefore processivity factors are also required to hold the DNA replicase on the template strand (1, 2).

Bacteriophage ϕ29 DNA polymerase is a protein-primed DNA-dependent replicase belonging to the eukaryotic-type family of DNA polymerases (family B). Other members of this family include polymerases with cellular, bacterial, and viral origins (3). ϕ29 DNA polymerase, like many other replicative polymerases, contains both 5′-3′ synthetic and 3′-5′ degradative activities within a single polypeptide chain. Its intrinsic insertion discrimination of 10^4 to 10^6 (4) is further improved 100-fold (5) through proofreading by the exonuclease domain. An extensive mutational analysis of ϕ29 DNA polymerase served to identify the catalytic residues required for these two activities, as well as those responsible for the stabilization of the primer terminus at the respective active sites; these residues are evolutionarily conserved in most DNA polymerases (reviewed in refs. 6 and 7). In addition, ϕ29 DNA polymerase shows three distinctive features compared with most replicases. First, it initiates DNA replication at the origins located at both ends of the linear genome by catalyzing the addition of the initial dAMP onto the hydroxyl group of Ser-232 of the bacteriophage terminal protein (TP), which acts as primer (reviewed in refs. 8–10). After a transition stage in which a sequential switch from TP priming to DNA priming occurs, the same polymerase molecule replicates the entire genome processively without dissociating from the DNA (11). Second, unlike ϕ29 DNA polymerase, most replicases rely on accessory proteins to clamp the enzyme to the DNA. These include thioredoxin in the case of T7 DNA polymerase (12, 13), the β-subunit of *Escherichia coli* PolIII holoenzyme (14), or the eukaryotic clamp protein, PCNA (15, 16). In contrast, ϕ29 DNA polymerase performs DNA synthesis without the assistance of processivity factors, displaying the highest processivity described for a DNA polymerase (>70 kb; ref. 11). A third distinctive property of ϕ29 DNA polymerase is the efficient coupling of processive DNA polymerization to strand displacement. This capacity allows the enzyme to replicate the ϕ29 double-strand genome without the need for a helicase (11). These two features, high processivity and intrinsic strand-displacement capacity, are the basis for the use of ϕ29 DNA polymerase in isothermal rolling circle amplification and whole genome amplification (17, 18).

The recently determined crystallographic structure of ϕ29 DNA polymerase has provided insights into the structural basis of both processivity and strand displacement in this small (66-kDa) replicase (19). A comparative analysis with the structure of other eukaryotic-type (family B) DNA polymerases, such as those from RB69 (20), *Thermococcus gorgonarius* (21), *Pyrococcus kodakaraensis* (22), *E. coli* (Protein Data Bank ID code 1Q8I), *Thermococcus* sp.9°N-7 (23), and *Desulfurococcus tok* (24), showed a common folding: a polymerization domain structured as a right hand containing the universal palm, fingers, and thumb subdomains, which form a groove in which primer-template DNA may be bound; and a 3′-5′ exonuclease domain having the residues involved in proofreading. The main difference between ϕ29 DNA polymerase and the above-mentioned family B DNA polymerases is the presence of two additional subdomains, both corresponding to sequence insertions specifically conserved in the protein-primed subgroup of DNA polymerases. These insertions are called TP regions (TPR), TPR1 and TPR2, initially described in refs. 25 and 26. Mutational analysis of TPR1 indicated its involvement in interactions with both TP and DNA substrates (25, 27). Although mutational data on TPR2 were unavailable, homology modeling of the DNA from the RB69 DNA polymerase ternary complex (28) onto the structure of ϕ29 DNA polymerase suggested possible functional roles. In particular, TPR2 helps to form a narrow tunnel around

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Abbreviations: TP, terminal protein; TPR2, TP region 2.

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the modeled downstream DNA, forcing the separation of the nontemplate strand from the template strand before its entry into the polymerase active site. Additionally, TPR2, along with the palm and thumb subdomains, forms a doughnut around the upstream duplex product, potentially enhancing processivity in a manner analogous to sliding-clamp proteins (19).

As presented here, biochemical analysis of a ϕ29 DNA polymerase deletion mutant lacking the TPR2 insertion directly demonstrates the functional role of this region in conferring both high processivity and strand-displacement capacity to the DNA polymerase.

Materials and Methods

Nucleotides and DNAs. [α-^{32}P]dATP [3,000 Ci/mmol (1 Ci = 37 GBq)] and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from Amersham Pharmacia. Unlabeled nucleotides were purchased from Amersham Pharmacia Biochemicals. Fifteen-mer oligonucleotide sp1 (5′-GATCACAGTGAGTAC) was 5′-labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase and purified electrophoretically on 8 M urea/20% polyacrylamide gels. Labeled sp1 was hybridized to oligonucleotides sp1c + 6 (21 mer) (5′-TCTATTGTACTCACTGTGATC) in the presence of 0.2 M NaCl and 50 mM Tris·HCl (pH 7.5), resulting in a primer/template construct that can be used in the coupled DNA polymerization/exonuclease, and to sp1c + 18 (33 mer) (5′-GGGGGGCCGCCGCCGCCGGTACTCACTGTGATC) to perform DNA-binding and processivity assays. Oligonucleotide D13 (5′-GCGGCGGCCCCCC), 5′-phosphorylated and complementary to the last 13 nucleotides of oligonucleotide sp1c + 18, was also hybridized to sp1/sp1c + 6 primer template to construct a 5-nt gapped structure with which to perform strand-displacement assays. Primers 45TPR2 (5′-CGCTAAATTCGCTAGTAACCCTACAAAAGACCCTGTTTATACACC) and 45TPR2c (5′-GGTGTATAAACGGGTCTTTTGTAGGGTTACTAGCGAATTTACCG) were designed to perform the mutagenesis reaction (see below).

Proteins. Phage T4 polynucleotide kinase was obtained from New England Biolabs. Wild-type ϕ29 DNA polymerase was purified from *E. coli* NF2690 cells harboring plasmid pJLPM (a derivative of pT7–4w2), as described (29). The ϕ29 DNA polymerase deletion mutant was purified essentially in a similar way, from *E. coli* BL21(DE3) cells harboring the corresponding recombinant plasmid.

Site-Directed Mutagenesis of ϕ29 DNA Polymerase. The ϕ29 DNA polymerase ΔTPR2 mutant was obtained by using the QuikChange site-directed mutagenesis kit provided by Amersham Pharmacia. Plasmid pJLPM containing the ϕ29 DNA polymerase gene was used as template for the mutagenesis reaction. Primers 45TPR2 and 45TPR2c are complementary and designed to hybridize to opposite strands of the plasmid flanking both sides of the region coding for residues Asp-398 to Glu-420, close to the ends of the TPR2 insertion. After temperature cycling using PfuTurbo DNA polymerase and treatment with DpnI endonuclease, synthesized DNA was transformed into XL1-blue supercompetent cells. The presence of the deletion and absence of other mutations were confirmed by sequencing the entire gene.

DNA Gel Retardation Assay. The interactions of either the wild-type or the ΔTPR2 mutant ϕ29 DNA polymerases with the primer-template oligonucleotides sp1/sp1c + 18 (15/33 mer) were analyzed. The incubation mixture contained, in a final volume of 20 μl, 12 mM Tris·HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulfate, 0.1 mg/ml BSA, 1.2 nM dsDNA (5′-labeled), and the indicated amounts of either wild-type or mutant ϕ29 DNA polymerase, in the presence of 1 mM $MnCl_2$. After incubation for 5 min at 4°C, the samples were subjected to electrophoresis in 4% (wt/vol) polyacrylamide gels (80:1, monomer/bis), containing 12 mM Tris-acetate (pH 7.5) and 1 mM EDTA and run at 4°C in the same buffer at 8 V/cm, essentially as described (30). After autoradiography, the ϕ29 DNA polymerase–dsDNA complexes were detected as a mobility shift (retardation) in the migrating position of the labeled DNA.

Polymerase/Exonuclease-Coupled Assay. The primer/template oligonucleotides sp1/sp1c + 6 (15/21 mer) contain a 6-nt-long 5′-protruding end, and therefore the primer strand can be used both as substrate for 3′-5′ exonuclease activity and for DNA-dependent DNA polymerization. The 12.5-μl incubation mixture contained 50 mM Tris·HCl (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT, 4% (vol/vol) glycerol, 0.1 mg/ml BSA, 1.2 nM 5′-labeled 15/21 mer, 24 or 360 nM wild-type or ΔTPR2 mutant ϕ29 DNA polymerases, respectively, and the indicated concentration of the four dNTP. After incubation for 10 min at 25°C, the reaction was stopped by adding EDTA up to a final concentration of 10 mM. Samples were analyzed by 8 M urea/20% PAGE and autoradiography. Polymerization or 3′-5′exonucleolysis is detected as an increase or decrease, respectively, in the size (15 mer) of the 5′-labeled primer.

The analysis of the base specificity during DNA-primed polymerization was studied by using four template/primer constructs (sp1/sp1c + 6), differing only in the first template base (position 16), and independent addition of each of the four dNTP at 100 μM. The reactions were performed as described above for the pol/exo-coupled assay but incubated on ice to reduce exonucleolytic degradation.

Processivity Assay. The processivity of the ΔTPR2 mutant ϕ29 DNA polymerase was analyzed at different enzyme/DNA ratios. The 12.5-μl incubation mixture contained 50 mM Tris·HCl (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT, 4% (vol/vol) glycerol, 0.1 mg/ml BSA, 1.2 nM 5′-labeled 15/33 mer, 50 μM dNTP, and the indicated decreasing amounts of either wild-type or ΔTPR2 mutant ϕ29 DNA polymerases. After incubation for 5 min at 25°C, the reactions were stopped by adding EDTA up to a final concentration of 10 mM. Samples were analyzed by 8 M urea/20% PAGE and autoradiography. Processivity of polymerization was assessed by analysis of the length of replication products under decreasing DNA polymerase/DNA ratios.

Strand-Displacement DNA Synthesis Assay. A primer/template molecule with a gap of 5 nt (see *Nucleotides and DNAs*) was used to study the strand-displacement capacity of the ΔTPR2 mutant of ϕ29 DNA polymerase. A primer/template construct (15/33 mer) that did not require strand displacement was also used as control. The 12.5-μl incubation mixture contained 50 mM Tris·HCl (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT, 4% (vol/vol) glycerol, 0.1 mg/ml BSA, 1.2 nM 5′-labeled 15/33 mer, 24 and 360 nM wild-type and mutant ϕ29 DNA polymerase, respectively, and the indicated concentration of the four dNTP. After incubation for 10 min at 25°C, the reaction was stopped by adding EDTA up to 10 mM. Samples were analyzed by 8 M urea/20% PAGE and autoradiography. The ability of the enzyme to carry out strand displacement was analyzed by comparing the length of the elongation products when using the gapped and the nongapped primer/template molecules.

Results

Deletion of TPR2, a Specific Insertion of Protein-Primed DNA Polymerases. ϕ29 DNA polymerase possesses two insertions in the palm subdomain, specifically conserved in the subgroup of DNA polymerases that use a protein as a primer (26). They are TPR1, whose conserved residues were proposed to make contacts with the TP and DNA (25, 27), and TPR2 with a biochemically

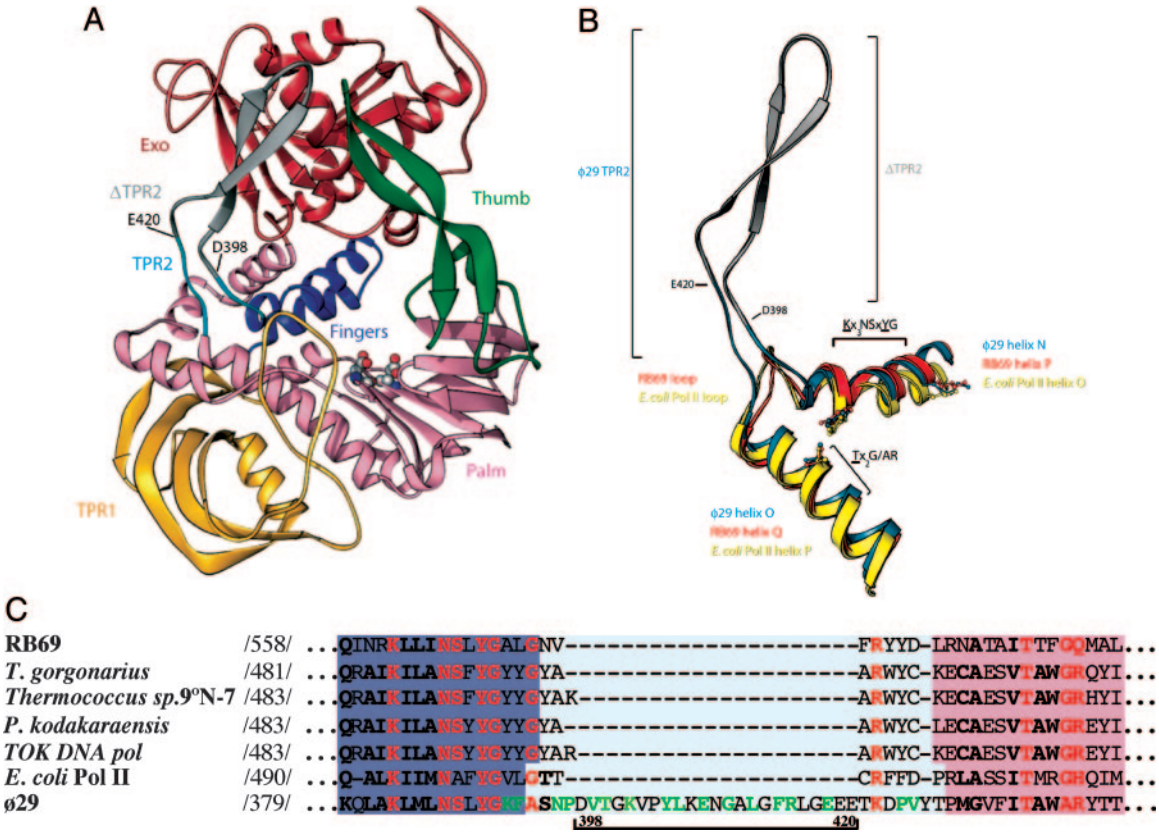

Fig. 1. ϕ29 DNA polymerase TPR2, a specific subdomain of protein-priming DNA polymerases. (*A*) Location of the TPR2 insertion of ϕ29 DNA polymerase. A ribbon representation of the eukaryotic-type ϕ29 DNA polymerase structure shows its four subdomains, colored as follows: 3′-5′ exonuclease domain in red, fingers in blue, palm in pink, and thumb in green. The ϕ29 DNA polymerase TPR2 insertion connecting helices N (fingers) and O (palm) is indicated in cyan, and the region deleted in the ΔTPR2 mutant is represented in gray. Crystallographic data are from ref. 19. (*B*) Superposition of the homologous α-helices from fingers and palm subdomains of the eukaryotic-type DNA polymerases from bacteriophages ϕ29 and RB69 and *E. coli* DNA polymerase II, obtained by automatic fitting of the conserved Lys and Tyr residues from motif Kx_3NSxYG and Thr residue from motif Tx_2G/AR by using the program SWISS-PDBVIEWER (www.expasy.org/spdbv). The ϕ29 DNA polymerase TPR2 insertion and its flanking α helices are colored in gray and blue, respectively. The corresponding helices of RB69 DNA polymerase and *E. coli* DNA polymerase II and the loop located between them are colored in red and yellow, respectively. Crystallographic data are from Protein Data Bank ID codes 1IG9 (RB69 DNA polymerase), 1XHX (ϕ29 DNA polymerase), and 1Q8I (*E. coli* DNA polymerase II). The amino acid side chains in ball-and-stick representation are the underlined amino acids in the Kx_3NSxYG and Tx_2G/AR motifs. (*C*) Amino acid sequence alignment of the region encompassing motifs Kx_3NSxYG to Tx_2G/AR of crystallized DNA-dependent DNA polymerases belonging to the eukaryotic-type (family B). The DNA polymerase nomenclature and sequences are compiled in ref. 3, with the exception of bacterial DNA polymerases from *T. gorgonarius* (GenBank accession no. P56689), *P. kodakaraensis* (GenBank accession no. BAA06142), *Thermococcus sp.*9°-N7 (23), DNA polymerase from the archaebacterial *D. tok* (GenBank accession no. 1QQCA), and DNA polymerase from bacteriophage RB69 (GenBank accession no. Q38087). The numbers indicate the position of the first aligned amino acid with respect to the N terminus of the respective DNA polymerase. Highly conserved residues among family B DNA polymerases are shown in red letters. Residues specifically conserved in the bacteriophage protein-primed subgroup of family B DNA polymerases are shown green. The amino acid sequence from residues Asp-398 to Glu-420, deleted in the ϕ29 DNA polymerase ΔTPR2 mutant, is indicated.

uncharacterized functional role (see Fig. 1*A*). The TPR2 insertion is formed by residues 394–427 and is located between the conserved motifs Kx_3NSxYG (motif B) and Tx_2G/AR (see Fig. 1 *B* and *C*). It contains a β-hairpin that connects α helices N (belonging to the fingers subdomain) and O (belonging to the palm subdomain) (ref. 19; see Fig. 1*B*) that is positioned just opposite the thumb, also structured as a β-hairpin in contrast to other DNA polymerases structurally solved. Both subdomains, together with the palm, acquire a doughnut-shaped conformation that could wrap the upstream DNA at the polymerization domain providing stability to the DNA polymerase/DNA complex during replication. At the same position, other DNA polymerases contain a short loop (see Fig. 1*B*), formed by seven to nine amino acid residues (see Fig. 1*C*). Therefore, as seen in the DNA polymerases aligned in Fig. 1*C*, the TPR2 insertion is present only in ϕ29 DNA polymerase but is conserved in the subgroup of protein-primed DNA polymerases, as described (26). To analyze the functional role of the TPR2 insertion, we constructed a ϕ29 DNA polymerase deletion mutant lacking amino acid residues Asp-398 to Glu-420. The resulting ϕ29 DNA polymerase mutant (ΔTPR2 mutant) maintains 11 amino acid residues between α helices N and O, to preserve the relative folding and orientation of the fingers with respect to the palm subdomain.

Removal of TPR2 Impairs ϕ29 DNA Polymerase DNA Binding. The modeling of a primer/template DNA onto ϕ29 DNA polymerase suggested that TPR2, together with the thumb, fingers, and palm subdomains, encircles the duplex DNA at the polymerization active site to confer processivity to the enzyme (19). To analyze the capacity of the ΔTPR2 mutant to efficiently bind a primer terminus, gel-shift assays were carried out by using a 15/33-mer hybrid molecule as substrate (see *Materials and Methods*). As shown in Fig. 2, the wild-type enzyme gives rise to a single shifted band, which we interpret as a stable complex competent for DNA replication (31), and whose intensity depends on the amount of enzyme added. The ΔTPR2 mutant was highly impaired in the formation of a stable complex with the DNA

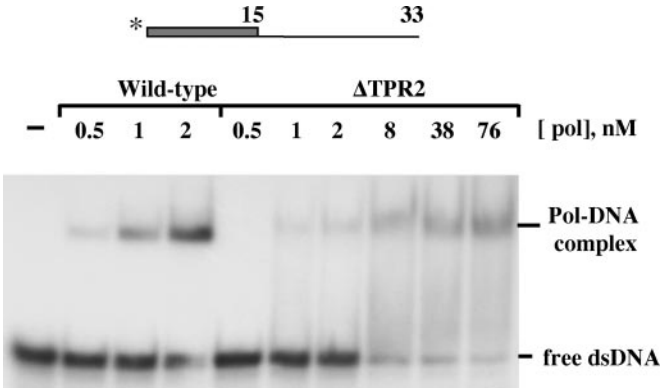

Fig. 2. ϕ29 DNA polymerase ΔTPR2 is impaired in its DNA-binding capacity. The assay was carried out as described in *Materials and Methods* by using a 5′-labeled 15/33 mer as substrate, in the presence of the indicated concentrations of wild-type or mutant ϕ29 DNA polymerases. Samples were analyzed by polyacrylamide gel electrophoresis and autoradiography. Bands corresponding to free DNA and to the DNA polymerase/DNA complex are indicated.

(Fig. 2). Moreover, in the presence of a relatively high concentration (8 nM) of ΔTPR2 mutant, the shifted band is smeared, indicating the formation of unstable DNA polymerase/DNA complexes.

The ϕ29 DNA Polymerase ΔTPR2 Mutant Displays Both Polymerization and Exonuclease Activities. To analyze both the 3′-5′ exonuclease and 5′-3′ polymerization activities of the mutant DNA polymerase, we studied the functional coupling between synthesis and degradation on a primer/template hybrid molecule as a function of dNTP concentration (see *Materials and Methods*). In the absence of nucleotides, the only bands that can be detected with the wild-type enzyme are primer degradation products due to the 3′-5′ exonuclease activity (see Fig. 3). As the concentration of the unlabeled dNTP provided is increased, this activity is progressively competed by the 5′-3′ polymerization, and net dNTP incorporation is observed as an increase in the size of the labeled primer; 100 nM dNTP is needed to completely outcompete the 3′-5′ exonuclease activity. Although the ΔTPR2 mutant yielded longer degradation products in the absence of nucleotides than did the wild-type enzyme, the mutant retained 3′-5′ exonuclease activity. The ΔTPR2 mutant also retained polymerization activity, although 500 nM dNTP was required to obtain an efficient elongation of the primer. However, at 20 nM dNTP, the +1 band was more intense than that obtained with the wild-type DNA polymerase. Moreover, the ΔTPR2 mutant also showed an improved capacity to incorporate the dNTP complementary to the last template position (compare both enzyme activities at 500 nM dNTP). These results could reflect a distributive behavior of the mutant DNA polymerase, unable to replicate further in the presence of such low dNTP concentration. On the other hand, the nucleotide insertion fidelity of the mutant during replication on primer/template constructs (see *Materials and Methods*) was similar to that of the wild-type DNA polymerase (data not shown).

That the ΔTPR2 mutant retained both exonuclease and polymerization activities together with a wild-type nucleotide insertion fidelity rules out the possibility of a general misfolding due to the deletion in the mutant polymerase.

ϕ29 DNA Polymerase ΔTPR2 Mutant Polymerizes Deoxynucleotides Distributively on Primer/Template Substrates. ϕ29 DNA polymerase is a paradigm for processive DNA replication, because it is able to incorporate >70 kb without dissociating from DNA in the absence of accessory factors (11). To study whether the

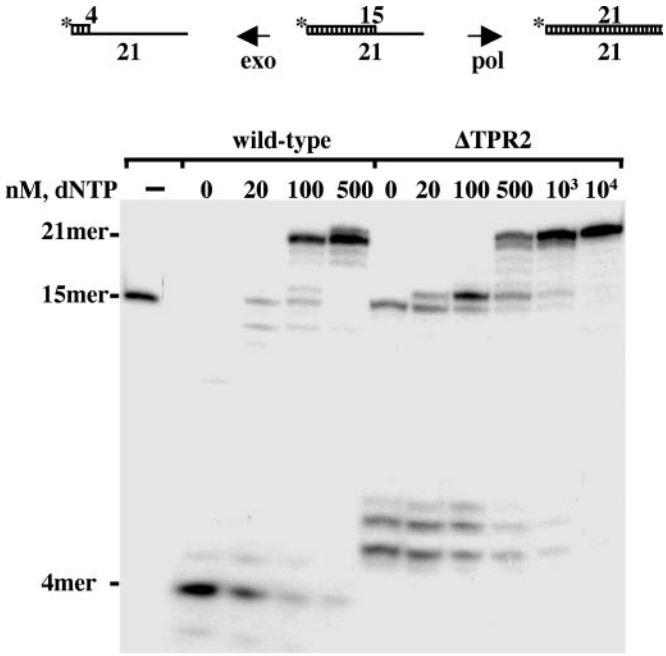

Fig. 3. The ϕ29 DNA polymerase ΔTPR2 mutant has both polymerization and exonuclease activities. The assay was carried out as described in *Materials and Methods* by using a ^{32}P-labeled 15/21 mer as primer/template DNA and the indicated concentrations of dNTP. Polymerase or 3′-5′exonuclease activities are detected as an increase or decrease, respectively, in the size (15 mer) of the 5′-labeled primer.

removal of the TPR2 insertion had any effect on processivity, we analyzed the chain length distributions during DNA polymerization by the ΔTPR2 mutant as a function of enzyme/DNA ratio. As shown in Fig. 4, decreasing the enzyme/DNA ratio did not alter the length (33 mer) of the elongation products made by the wild-type enzyme up to a limit in which the ratio was too low to detect primer elongation. Conversely, the length of the products synthesized by the ΔTPR2 mutant decreased with the enzyme/DNA ratio (Fig. 4), in agreement with a distributive polymerization pattern.

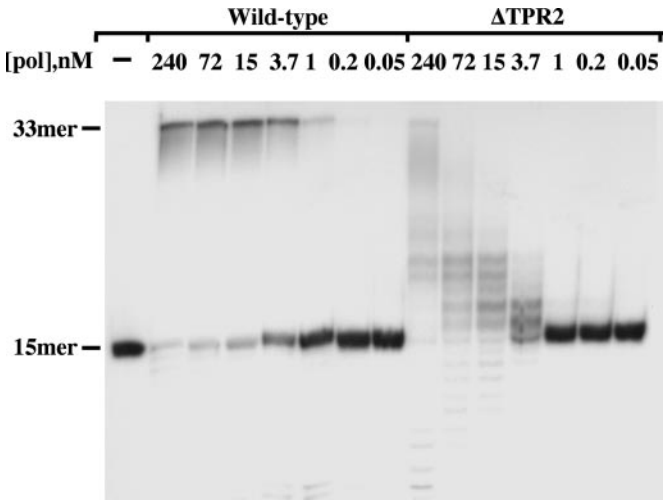

Fig. 4. The ϕ29 DNA polymerase ΔTPR2 mutant shows a distributive polymerization pattern. The assay was carried out as described in *Materials and Methods* by using a 5′-labeled 15/33 mer as substrate, in the presence of the indicated concentrations of wild-type or mutant ϕ29 DNA polymerases.

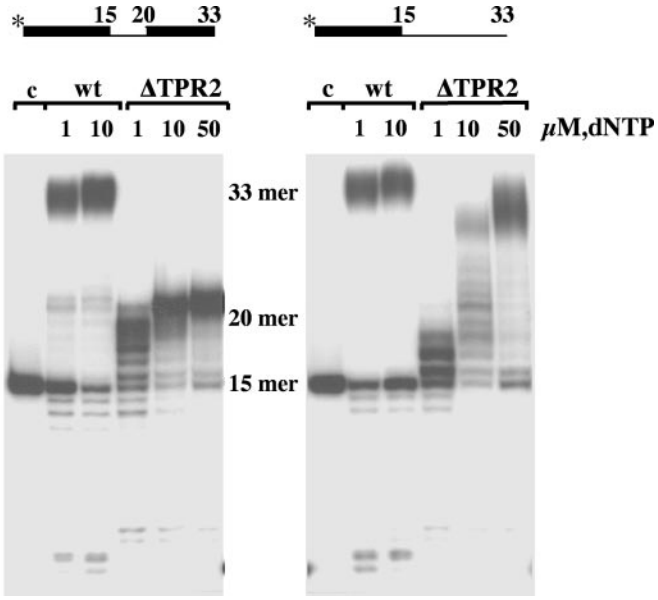

Fig. 5. Removal of the TPR2 insertion disables DNA polymerization coupled to strand displacement. The polymerization assay was carried out on a 5-nt gapped and nongapped substrate as described in *Materials and Methods* by using 24 or 360 nM wild-type or mutant DNA polymerases, respectively, and the indicated increasing concentration of the four dNTP. After incubation for 10 min at 25°C, the reaction was stopped, and samples were analyzed by 8 M urea/20% PAGE and autoradiography.

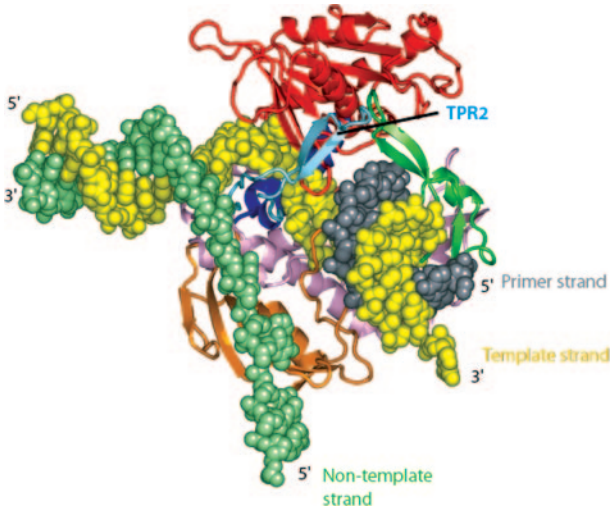

Fig. 6. Modeling processivity and strand displacement in ϕ29 DNA polymerase. Based on the results presented here and on the crystallographic structure of ϕ29 DNA polymerase (19), the TPR2 insertion would contribute to a full encirclement of the DNA substrate, conferring a remarkable processivity, and also acts as a structural barrier, which would force the DNA strands of the parental DNA to diverge (melt). Because ϕ29 DNA polymerase translocates after each polymerization cycle, the TPR2 subdomain would act as a wedge to couple polymerization to strand displacement. ϕ29 DNA polymerase subdomains are colored as indicated in Fig. 1*A*. Modeled DNA is colored as follows: growing primer strand in gray, template strand in yellow, and displaced strand in green.

TPR2 Is Required for the Strand-Displacement Activity of ϕ29 DNA Polymerase. To analyze whether the TPR2 deletion had any consequence in the strand-displacement capacity of the DNA polymerase, we analyzed the extent of primer elongation on gapped DNA molecules (see Fig. 5). As expected, wild-type ϕ29 DNA polymerase was able to fill the gap (5 nt), continuing DNA synthesis through the duplex region via strand displacement. There is only a faint band surrounding positions +6 to +7, where the dsDNA region starts, indicating that the polymerase only rarely failed to make the transition from gap filling to strand displacement-coupled synthesis. However, the ΔTPR2 mutant was very inefficient at this transition; it failed to extend most primers after filling the 5-nt gap. The TPR2 insertion therefore appears to be crucial for polymerization coupled to strand displacement. In a parallel control experiment, we used the primer/template molecule P15/T33 (see Fig. 5), which contains the same template strand but lacks a downstream nontemplate oligonucleotide. The wild-type enzyme was able to fully extend the primer at a low dNTP concentration (1 μM); the ΔTPR2 mutant polymerase was also able to fully extend the primer, although at a higher dNTP concentration. That the ΔTPR2 mutant polymerase did not show a preferential stop at positions +6 to +7 rules out the possibility that the blockage observed in the gapped DNA was because of sequence context. The above experiments confirm the hypothesis that the TPR2 insertion is required for strand displacement by ϕ29 DNA polymerase.

Discussion

ϕ29 DNA polymerase replicates the entire ϕ29 double-stranded linear genome in the absence of processivity factors and DNA helicases (11). Such an enzymatic potential relies on the intrinsic processivity and strand-displacement capacity of the DNA polymerase. One of the most intriguing aspects of ϕ29 DNA polymerase is how this relatively small enzyme is able to coordinate both features in the same polypeptide chain.

The recently determined structure of ϕ29 DNA polymerase suggested a functional role for the specific insertion TPR2, present in the subfamily of protein-primed DNA polymerases. This insertion constitutes a linker region between the fingers and palm subdomains, connecting α helices N (belonging to the fingers) and O (belonging to the palm; ref. 19). The doughnut-shaped structure formed by TPR2, thumb, and palm subdomains appears to encircle the upstream DNA at the polymerization domain during replication, providing stability to the DNA polymerase/DNA complex. Additionally, the passage of downstream template through a narrow pore before entering the active site could force the separation of template and nontemplate strands, as well as further stabilize the polymerase/DNA complex (19).

A ϕ29 DNA polymerase deletion mutant lacking most of the TPR2 insertion displayed a ratio between polymerization and 3′-5′ exonuclease activities close to that of the wild-type enzyme, although high mutant DNA polymerase/DNA ratios were required for activity. This, together with the poor stability of the ΔTPR2 mutant/DNA complexes in gel-shift assays, indicates that the TPR2 insertion is required by the DNA polymerase to maintain strong DNA binding. A direct consequence of the reduction in DNA-binding capacity was the loss of the extraordinary processivity of ϕ29 DNA polymerase, reducing the number of nucleotides incorporated per binding event from >70,000 to only a few. The results support the hypothesis that the TPR2 insertion is a processivity-enhancing subdomain.

The polymerization domains of DNA polymerases can be described, by analogy to a right hand, to contain palm, fingers, and thumb subdomains. Together, these subdomains form a groove in which primer-template DNA is bound. Comparison of apo with DNA-bound polymerase structures often shows an inward rotation of the thumb subdomain in the presence of oligonucleotide (28, 32–34). Although such conformational changes stabilize DNA binding, most replicative polymerases require additional accessory sliding clamp factors to achieve the

processivity required for genome duplication (14, 15, 35–38). These proteins have a toroidal conformation with a hole in the center that encircles DNA, tethering the DNA polymerase to the primer-terminus junction to ensure high processivity. ϕ29 DNA polymerase is intrinsically processive, because the TPR2 insertion, together with a specialized thumb, fingers, and palm subdomains, constitutes an internal clamp (19) to provide the enzyme with the maximal DNA-binding stability required to replicate the entire genome (19,285 bp) from a unique DNA polymerase-binding event.

Of interest, removal of the TPR2 insertion also abolishes the capacity of ϕ29 DNA polymerase to couple polymerization to strand displacement. The ϕ29 DNA polymerase structure shows that the TPR2 insertion, together with the fingers, palm, and exonuclease domain, forms a tunnel whose narrow dimensions permit binding of only a single-stranded DNA template chain, in comparison with the open channel described in other family B DNA polymerases (19). A consequence of this topological restriction is that only the template strand of the dsDNA genome can thread through the tunnel to reach the polymerase active site. Although we cannot rule out that other subdomains can contribute to the strand-displacement capacity of the polymerase, the results presented here validate the proposed key role of the TPR2 insertion in such a capacity (19): it could act as a molecular "wedge" to separate the parental DNA strands, thus conferring a helicase-like function on the DNA polymerase (Fig. 6). In fact, the region responsible for dsDNA unwinding must be located very close to the polymerization active site, because the ΔTPR2 mutant stops replication where the duplex region starts. Similar examples of a dsDNA intercalating structure have been described in several RNA polymerases such as those from bacteriophages T7 (39) and ϕ6 (40). In these cases, the polymerase can unwind the dsRNA and perform successive strand-separation reactions in the absence of a helicase during the initiation steps of transcription. Whether the TPR2 insertion merely represents a steric hindrance to force the unwinding of dsDNA, or, on the contrary, plays an active role in such a helicase-like activity involving specific residues remains to be elucidated.

Conclusion

ϕ29 DNA polymerase has evolved to solve two crucial requirements of genome replication, processivity, and strand displacement by inserting an amino acid sequence region (TPR2) between the fingers and palm subdomains. This insertion, which is common to all protein-primed DNA polymerases, closes the universally conserved dsDNA groove in the polymerization domain and generates both an internal clamp and a tunnel that can mimic a helicase by encircling the single-stranded template. Therefore, the helicase and clamp-like features conferred by TPR2, first characterized here for ϕ29 DNA polymerase, are likely to be evolutionarily conserved among other members of protein-primed DNA polymerases.

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